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No differences in benzo(a)pyrene hydroxylase activity in the human immature placenta and in the human fetal liver from cigarette smoking and nonsmoking women*)

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Cigarette smoking during pregnancy has been shown to increase the incidence of abortions, premature deliveries and lighter weight of the newborns. However, an explicit explanation for these findings is still lacking and until now it cannot be excluded that the constitution of the pregnant woman who smokes might be related to these observations and not to the smoking habit per se [2, 5, 11, 24, 32, 33]. Besides these clinical findings it was demonstrated that in the term placenta the **enzymatic hydroxylation of benzo(a)pyrene (BP), one of several carcinogens present in cigarette smoke, was found in almost all placentas from women with smoking habits during pregnancy** but no or only little BP hydroxylase could be detected in the placentas from nonsmokers [13, 29, 30]. The relationship of the clinical findings and the ability of components of cigarette smoke to stimulate BP hydroxylation by enzymes in the placenta still remains to be clarified.

The results of these studies have prompted our laboratory in late 1970 to evaluate whether in the immature placenta BP hydroxylase activity could be detected and whether there exists a correlation between the activity of this enzyme and the habit of cigarette smoking during the early phase of pregnancy. Since then, data were published showing that in the immature placenta the capacity to metabolize BP was not found in the placentas from nonsmokers, but the placentas from smokers metabolized BP, though being lower in the immature than in the term tissue. However, no enzyme activity was detectable in the placenta from smokers before the eleventh week of pregnancy [7, 19]. Similarly, in rats the maximum stimulatory effect by BP treatment on BP hydroxylase in the placenta was not achieved before day 15 of gestation [26, 27]. Besides the above mentioned data, the capacity of enzymes in the human placenta to metabolize drugs and foreign compounds is rather limited. Immature or mature placentas were inactive in metabolizing hexobarbital, chlorpromazine, codein, aniline, aminopyrine, amphetamine, p-nitrobenzoic acid [8], p-nitroanisole, acetaniline, amphetamine, aniline [3], phenobarbital [29] though N-methylaniline [20] and neoprontisil [8] were found to be metabolized and 3-methyl-4-monomethylamino-

Curriculum vitae

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azobenzene only in placentas from cigarette smokers [29].

In addition, we investigated whether the fetal liver is able to hydroxylate BP and to N-demethylate ethylmorphine. In several laboratories the existence of drug metabolizing enzyme activity in the fetal liver has been demonstrated [6, 15, 16, 17, 18, 19, 20, 21, 23, 31]. So far, the substrates found to be metabolized by fetal liver enzymes include: Benzo(a)pyrene, N-methylaniline, chlorpromazine, p-nitrobenzoic acid, aniline, aminopyrine, hexobarbital [16, 17, 18, 19, 20, 21] benzo(a)pyrene, ethylmorphine, aniline [23], laurate and testosterone [31]. The latter authors were unable to detect any aminopyrine N-demethylation and benzo(a)pyrene hydroxylation though they demonstrated the presence of all components required for the oxidation of drugs, steroids, and foreign compounds. Furthermore, no demethylation activity for p-nitroanisol and N-monomethyl p-nitroaniline could be detected [22].

This report demonstrates that in the immature placenta the level of BP hydroxylase activity does not differ between the smoking and nonsmoking group and that the fetal liver is capable of metabolizing BP and ethylmorphine.

1 Materials and methods

1.1 Tissues:

Placenta and fetal liver were obtained from patients undergoing therapeutic abortion for socio-medical reasons. All patients received the following premedication and anesthesia: Cyclobarbital, 100 mg; isothipendyl, 20 mg; fentanyl citrate, 0.10 mg; droperidol, 5.0 mg; atropin, 0.25 mg; succinyldicholine, 100—200 mg; thiopental, 150 mg; and halothane, nitrous oxide, oxygen, and 300—500 ml of a plasma expander and 300—500 ml of a 5% glucose solution. Clinical histories were recorded by staff members of the hospital while one author (E. S.) asked the women for smoking habits during pregnancy. The duration of pregnancy was estimated from the last menstrual period. All patients lived in Berlin. Tissues were obtained from 5 patients by hysterotomy and from 13 patients by curettage. The tissues obtained during surgery were immediately frozen in liquid nitrogen and stored in liquid nitrogen until use within 1—5 days. The tissues were than thawed at room temperature. Two 8 gram samples of one placenta were dissected from connective tissue. The placenta was carefully minced and homogenized in ice cold 0.32 M sucrose-tris HCl solution (0.01 M tris HCl, pH 7.4). The whole fetal liver was homogenized in the same solution. Only whole homogenates were employed for the enzyme assays.

1.2 Enzyme assays:

The following incubation mixture was used for the measurement of benzo(a)pyrene hydroxylase activity: 5 enzyme units of glucose-6-phosphate dehydrogenase in 0.05 M tris HCl buffer (pH 7.4); glucose-6-phosphate in 0.05 M tris HCl buffer (pH 7.4), 6,5 μmoles; nicotine adenine dinucleotide phosphate (NADP), 0.5 μmoles; nicotine adenine dinucleotide (NAD), 0.6 μmoles; adenosine triphosphate (ATP), 2.01 μmoles; NADP, NAD, and ATP were dissolved in 0.1 M KH_2PO_4—K_2HPO_4 buffer (pH 7.4), nicotinamide, 12 μmoles; KCl, 200 μmoles; $MgCl_2$, 10 μmoles; 1.0 ml KH_2PO_4—K_2HPO_4 buffer (pH 7.4), benzo(a)pyrene 6.39×10^{-5} M dissolved in 0.1 ml acetone, and 0.5 ml of placental homogenate equivalent 100—200 mg wet weight or 0.5 ml fetal liver homogenate equivalent 10—20 mg wet weight. The total volume was 3.1 ml. Placental homogenates were incubated for 20 min at 37°C and liver homogenates for 10 min at 37°C.

For the measurement of ethylmorphine N-demethylase each incubation flask consisted of the following: 3 enzyme units of glucose-6-phosphate dehydrogenase, glucose-6-phosphate, 20 μmoles; NADP, 2 μmoles; nicotinamide, 20 μmoles; $MgCl_2$, 10 μmoles; semicarbacide HCl, 37.5 μmoles; KH_2PO_4—Na_2HPO_4 buffer, pH 7.4, 200 μmoles; 1.0 ml of fetal liver homogenate equivalent to 400 mg wet weight; ethyl-morphine, 2×10^{-3} M dissolved in 1.0 ml 1.15% KCl, and 1.15% KCl solution to give a final volume of 5.0 ml. The reaction mixtures were incubated for 15 min at 37°C.

For each enzyme assay two blanks were employed. One blank was incubated with the complete reaction mixture but without the homogenate and with the substrate. A second blank was incubated with the homogenate but without the substrate. Under these experimental conditions enzyme activities were linear.

1.3 Analytical procedures:

Benzo(a)pyrene hydroxylase activity was assayed as detailed previously [9, 28]. The hydroxylated metabolite(s) of benzo(a)pyrene produced during the incubation was measured with a Zeiss fluorometer, using 3-hydroxybenzo(a)pyrene as a reference. Ethylmorphine N-demethylase activity was measured by the Nash procedure [12] as described in the literature [1] with the exception that 1.0 ml 50% trichloroacetic acid was added to stop the reaction.

2 Results

2.1 Enzymatic hydroxylation of benzo(a)-pyrene in the placenta

The hydroxylation rate of benzo(a)pyrene was measured in the placentas obtained from 9 nonsmokers and from 9 smokers. In both groups the mean gestational age did not vary consid-

Tab. I. Benzo(a)pyrene hydroxylase in the immature placenta and benzo(a)pyrene hydroxylase and ethylmorphine N-demethylase in the fetal liver.

Patient	Age (years)	Duration of pregnancy (weeks)	Cigarettes smoked daily	Placenta BP Hydroxylase (μg Hydroxy. BP/g/h) Mean	Range	Fetal liver BP Hydroxylase (μg Hydroxy-BP/g/h)	Ethylmorphine N-Demethylase (μg HCOH/g/h)
nonsmokers							
E. S.	28	10	0	0.392	0.174—0.609	—	—
G. G.	39	12	0	0.094	0.082—0.105	—	—
H. M.	32	12	0	0.180	0.180—0.180	4.007	597.00
A. B.[1])	24	12	0	<0.050		—	—
J. K.	33	14	0	<0.050		—	—
C. D.[2])	34	14	0	1.510	1.510—1.510	—	—
H. K.	31	14	0	<0.050		—	—
R. F.	31	14	0	0.088	0.060—0.116	10.000	722.70
G. H.	33	18	0	<0.050		3.940	153.00
Mean ± S. D.	32±4	13±2		0.252±0.489			
smokers							
R. A.	37	14	3—4	<0.050		—	—
E. W.	35	14	3—5	0.441	0.347—0.534	—	—
B. D.	20	12	3—5	0.068	0.068—0.068	—	350.40
G. G.[3])	40	10	8—10	0.379	0.208—0.550	—	—
D. M.	27	12	10—12	<0.050		—	—
R. J.	35	12	10—20	<0.050		—	—
H. K.[4])	42	16	20	0.812	0.730—0.894	0.656	435.00
X. G.[5])	19	10	20	1.176	0.915—1.436	—	—
R. S.	32	12	25	0.228	0.228—0.228	—	—
Mean ± S. D.	32±8	12±2		0.345±0.413			

Five patients received the following daily medications during pregnancy:
[1]) A. B.: ethinyl estradiol, 0.05 mg or ethinyl estradiol, 0.05 mg and 17α-ethinyl-19-nortestorone-acetate, 2.00 mg
[2]) C. D.: Barbital, 300 mg; Phenobarbital, 30 mg; Aminophenazone, 18 mg; Phenacetin, 200 mg; Acetylsalicylic acid, 250 mg; Coffein, 50 mg
[3]) G. G.: Trifluoperazine, 2.36 mg; trans-2-phenylcyclopropylaminsulfat, 27.4 mg; Methaqualone, 250 mg; Diphenhydramin, 25 mg; Cyproheptadine, 12 mg
[4]) H. K.: Diphenylhydantoin, 105 mg; Phenobarbital, 53 mg; Coffein, 90 mg; Sodiumbromide, 300 mg; Ammoniumbromide, 75 mg
[5]) X. G.: Fluphenazine, 2.0 mg.
For experimental details see Materials and Methods; — = not investigated

erably, being 13 and 12 weeks, respectively. The results of these studies are shown in Tab. I.

In the placentas obtained from nonsmokers only in four no BP hydroxylase activity could be detected, i. e. incubation of the placenta with BP did not result in fluorescence exceeding the blank values. One of the patients (A. B.) accidently received an oral contraceptive. However, in five of the placentas from nonsmokers BP hydroxylase activity was detectable. The degree of BP hydroxylation by enzymes in the placenta varied 17 fold, ranging from 0.088 μg 3-Hydroxy-BP/g tissue/hr (Patient R. F.) to 1.510 μg 3-Hydroxy-BP/g tissue/hr (Patient C. D.). The highest enzyme activity was measured in the placenta of Patient C. D. who was taking high doses of barbiturates, phenacetin and other drugs before and during pregnancy. The mean value of enzyme activity in the placentas from nonsmokers was 0.252 ± 0.489 μg 3-Hydroxy-BP/g tissue/hr.

In the placentas from 9 smokers in 3 no metabolism of BP was found (patients R. A., D. M., R. J.). These women smoked 3 to 4 or more than 10 cigarettes daily. In the other 6 placentas the rate of BP hydroxylation varied and no corre-

lation was found between the daily number of cigarettes smoked and the level of BP hydroxylase activity. In this group the mean BP hydroxylase activity was $0.344 \pm 0.413\,\mu g$ 3-Hydroxy-BP/g tissue/hr.

In three placentas obtained during the eleventh week of pregnancy (from nonsmokers E. S. and from smokers G. G. and X. G.) BP hydroxylase activity was detectable.

With the exception of placentas from nonsmokers C. D. and H. M. and from smokers B. D. and R. S. the rate of BP hydroxylation was not identical in the two samples of one placenta examined. This indicates that this enzyme might be predominantly localized in distinct areas of the placenta.

2.2 Enzymatic hydroxylation of benzo(a)-pyrene and enzymatic N-demethylation of ethylmorphine in the fetal liver

The age of the fetuses obtained from 3 nonsmokers and 2 smokers ranged from 12 to 16 weeks. The results (Tab. I) demonstrate the **existence of the two metabolizing enzyme reactions studied in all liver preparations,** though large variations were found in the hydroxylation rate of BP and the rate of ethylmorphine N-demethylation. Though only a small number of fetal livers was investigated, **probably no relationship exists between the level of enzyme activities and fetal age and the level of BP hydroxylase activity in the placenta and fetal liver.**

3 Discussion

The results presented in this paper do not demonstrate differences in the levels of BP hydroxylase activity in the placenta obtained from nonsmoking and cigarette smoking women between the eleventh and nineteenth week of pregnancy. This might suggest that components of cigarette smoke have no or only a small stimulatory effect on the BP hydroxylation rate during this phase of placental development. The **differences between our results and those reported in the literature** [6, 19] can be related to the following factors: a) The methods used for measuring BP hydroxylase activity were slightly different. In addition, in our experiments the immediate storage of the placenta in liquid nitrogen may prevent the degradation of a labile form of BP hydroxylase. b) The women of the nonsmoking group might have been exposed to foreign compounds or drugs that resulted in elevated levels of BP hydroxylase activity. Interestingly, the highest enzyme activity is found in the placenta of nonsmoker C. D. who was taking high doses of drugs. Some of these drugs (barbiturates and probably aminophenazone) do stimulate various drug metabolizing enzyme reactions in man [4]. However, it has not been established that they enhance the hydroxylation of BP. c) Other factors involved may be genetic differences in the capacity to metabolize BP and furthermore variations in the living conditions.

The disproportionate distribution of this enzyme found in our experiments indicates a distinct localization within differentiated parts of the placenta. Recently, regional differences in the steroid biosynthesis in the mature placenta could be related to specific morphological structures [10].

Independent of the smoking habits of the mother BP hydroxylase activity was found in all fetal livers investigated. The capacity of the fetal liver to metabolize BP and other chemicals does not necessarily imply a detoxification mechanism. Metabolites, produced in the fetal tissues and otherwise safe for adult tissues, might be harmful for the rapidly proliferating and differentiating fetal tissues. In addition, highly reactive intermediates, as for example epoxides deriving from the metabolism of BP and other chemicals might not be converted rapidly enough to protect fetal tissues susceptible to the cytotoxic effects of these compounds [14].

In conclusion, more work is needed to evaluate the relevance of the drug metabolizing capacity of fetal tissues, including the placenta. In addition, alterations in maternal drug metabolism may occur in those women who are exposed to chronic drug administration during pregnancy and thus might affect fetal development. Under experimental conditions, chronic phenobarbital treatment of pregnant rats showed markedly decreased drug metabolizing enzyme activity when compared with nonpregnant phenobarbital treated rats [25].

Summary

Benzo(a)pyrene (BP) hydroxylase activity was assayed in the immature placenta obtained from 9 nonsmoking and from 9 cigarette smoking women undergoing therapeutic abortion for socio-medical reasons between the eleventh and nineteenth week of pregnancy. In the nonsmoking group the activity of this enzyme was not detectable in 4 placentas. In the other 5 placentas the hydroxylation rate of BP ranged from 0.088 to 1.510 μg 3-Hydroxy-BP/g tissue/h **(Tab. I).** In the placentas obtained from 9 women with a history of cigarette smoking 3 had no BP hydroxylase activity. These women smoked 3 to 20 cigarettes daily. The other 6 women smoked almost the same daily number of cigarettes and in the placentas of these individuals the rate of BP hydroxylation ranged from 0.068 to 1.176 μg 3-Hydroxy-BP/g tissue/h. The mean $\pm$ S. D. of BP hydroxylase activity was 0.252 $\pm$ 0.489 and 0.345 $\pm$ 0.413 μg 3-Hydroxy-BP/g tissue/h in the nonsmoking and cigarette smoking group, respectively. These data suggest that between the eleventh and nineteenth week of pregnancy cigarette smoke has no or only a small stimulatory effect on placental BP hydroxylase activity. Furthermore, other factors than components of cigarette smoke might enhance enzymatic BP hydroxylation. For example, the highest enzyme activity (1.510 μg 3-Hydroxy-BP/g tissue/h) was found in the placenta of a nonsmoker who was taking high doses of barbiturates and other drugs. — The disproportionate distribution of this enzyme found in our experiments indicates a distinct localization within differentiated parts of the placenta.

In the fetal liver the activity of enzymes that hydroxylate BP and N-demethylate ethylmorphine was demonstrated. No correlation was observed between the levels of BP hydroxylase activity in the fetal liver and in the placenta.

In fetal tissues the capacity to metabolize drugs and foreign compounds does not necessarily imply a detoxification mechanism since metabolites derived from these reactions might be harmful for the rapidly proliferating and differentiating fetal tissues.

Keywords: Benzo(a)pyrene hydroxylase, cigarette smoking, human immature placenta, human fetal liver, drug metabolism.

Zusammenfassung

Keine Unterschiede der Aktivitäten von Benzo(a)pyren-Hydroxylase in der unreifen menschlichen Plazenta und in der fetalen Leber bei Raucherinnen und Nichtraucherinnen.

Bei 18 Frauen, 9 Nicht- und 9 Zigarettenraucherinnen, wurde eine Schwangerschaftsunterbrechung aus sozialmedizinischen Gründen zwischen der elften und neunzehnten Woche durchgeführt. Die Benzo(a)pyren (BP)-Hydroxylase Aktivität wurde in der Plazenta aller Frauen bestimmt. In der Gruppe der Nichtraucherinnen war in 4 Plazenten die Aktivität dieses Enzyms nicht nachweisbar, während in 5 Plazenten die BP Hydroxylierungsrate 0,088 bis 1,510 μg 3-Hydroxy-BP/g Gewebe/Std. betrug **(Tab. I).** In der Gruppe der Raucherinnen, die täglich 3 bis 20 Zigaretten rauchten, war in 3 Plazenten die Aktivität der BP Hydroxylase nicht meßbar. Bei 5 Patientinnen variierte die Hydroxylierungsrate in der Plazenta von 0,068 bis 1,176 μg 3-Hydroxy-BP/g Gewebe/Std. Der Mittelwert ($\pm$ S. D.) der BP Hydroxylaseaktivität betrug 0,252 $\pm$ 0,489 μg 3-Hydroxy-BP/g Gewebe/Std. in der Gruppe der Nichtraucherinnen gegenüber 0,345 $\pm$ 0,413 μg 3-Hydroxy-BP/g Gewebe/Std. in der Gruppe der Raucherinnen. Diese Daten zeigen, daß während der elften bis neunzehnten Woche der Schwangerschaft das Rauchen von Zigaretten keine oder nur eine geringfügig stimulierende Wirkung auf die plazentare BP Hydroxylaseaktivität hat. Da die höchste Enzymaktivität (1,510 μg 3-Hydroxy-BP/g Gewebe/Std.) in der Plazenta einer Nichtraucherin gefunden werden konnte, die hohe Dosen an Barbituraten und anderen Arzneimitteln eingenommen hatte, muß angenommen werden, daß außer den Komponenten des Zigarettenrauches auch Arzneimittel die BP Hydroxylierungsrate erhöhen können. — Die ungleichmäßige Verteilung der BP Hydroxylaseaktivität in der Plazenta läßt eine spezifische Lokalisation dieses Enzyms innerhalb gewisser morphologischer Strukturen vermuten.

In der fetalen Leber war sowohl die Hydroxylierung von BP als auch die N-Demethylierung von Äthylmorphin nachweisbar. Zwischen der Höhe der BP Hydroxylaseaktivität in der fetalen Leber und in der Plazenta konnte keine Korrelation gefunden werden.

Die Fähigkeit der fetalen Gewebe, Arzneimittel und Fremdstoffe zu metabolisieren, kann nicht zwangsläufig als Schutzmechanismus gedeutet werden. Ein Einfluß der Metaboliten auf embryonale Wachstums- und Differenzierungsprozesse ist nicht auszuschließen.

Schlüsselwörter: Arzneimittelstoffwechsel, Benzo(a)pyren Hydroxylase, menschliche fetale Leber, menschliche unreife Plazenta, Zigarettenrauchen.

Résumé

Similitude d'activite de l'hydroxylase du benzopyrène au niveau du placenta humain immature et du foie foetal chez les fumeuses et les non fumeuses

Nous avons évalué l'activité d'hydroxylation du Benzopyrène (BP) chez neuf fumeuses et 9 non fumeuses ayant subi un avortement thérapeutique entre la 11ème et la 19ème semaine de grossesse. Chez les non fumeuses, l'activité de cette enzyme ne put être mise en évidence dans 4 placentas. Dans les 5 autres placentas, le taux d'hydroxylation du BP fut compris entre 0,088 et 1,510 μg de 3-Hydroxy-Benzopyrène par gramme de tissu et par heure **(Tab. I).** Chez les fumeuses 3 placentas ne montrèrent

aucune activité d'hydroxylation du BP (ces femmes fumaient de 3 à 20 cigarettes par jour). Chez les 6 autres femmes, qui fumaient le même nombre de cigarettes par jour, on trouva une activité d'hydroxylation placentaire du BP comprise entre 0,068 et 1,176 μg de 3-Hydroxy-BP par gramme de tissu par heure. L'activité d'hydroxylation moyenne $\pm$ un écart type fut respectivement de 0,252 $\pm$ 0,489 μg de 3-OH-BP/g de tissu/heure chez les non fumeuses et de 0,345 $\pm$ 0,413 μg de 3-OH-BP/g de tissu/heure chez les fumeuses.

Ces faits suggèrent que la tabagisme a peu ou pas d'influence sur l'activité hydroxylasique placentaire entre la 11ème et la 19ème semaine de gestation.

Bien plus, d'autres facteurs que les composants de la fumeé de cigarettes peuvent augmenter l'hydroxylation du Benzopyrène. Par exemple: le taux le plus haut d'activité (1,510 μg 3-OH-BP/g de tissu/heure) fut trouvé chez une non fumeuse prenant de fortes doses de barbituriques et d'autres drogues.

La distribution variable de cette enzyme dans nos expérimentations montre une localisation particulière de l'enzyme d'un endroit à l'autre du placenta.

Nous avons mis en évidence dans le foie foetal, une activité enzymatique qui provoque l'hydroxylation du BP et du N-demethylate d'éthylmorphine. Nous n'avons pas trouvé de différence significative d'activité enzymatique de la BP-hydroxylase entre le foie foetal et la placenta.

Dans les tissus foetaux, la capacité de métaboliser les drogues et les composés étrangers n'implique pas nécessairement un mécanisme de détoxication vu que les catabolites de telles réactions pourraient être nuisibles à la croissance et à la différenciation des tissus foetaux.

Mots-clés: Benzopyrène hydroxylase, foie foetal, métabolisme des drogues, placenta humain immature, tabagisme.

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